SOME EXPERIENCES WITH RED-DIODE LASER (630 nm) EXCITABLE DYES ON THE FLOW CYTOMETER

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ABSTRACT

We investigated the use of a cytometer equipped with a red diode laser as a possible detector for biological warfare agents (Becton-Dickinson FACSCalibur™ equipped with a red diode laser in addition to the Argon laser). These instruments are smaller, require less power, and also have a reduced logistics burden. We decided to focus our efforts on YOYO-3, TOTO-3, and TOPRO-3, since we have worked with the blue excitable analogs of these dyes. Our initial studies with YOYO-3 and TOTO-3 showed little, if any, staining of the bacteria. A possible explanation is that these dyes are probably too large to properly intercalate with the DNA without some prior treatment. TOPRO-3, on the other hand, is a much smaller molecule than either of the dimeric cyanine dyes that were evaluated. Consequently, we were able to obtain histograms with both vegetative and spore-forming bacteria. The differences in the histograms showed that we could readily differentiate between spore-forming and vegetative bacteria. The background signals obtained with this dye were also significantly lower than those that we observed with either the blue or green excitable dyes. We then used this dye as a detector during Joint Field Trial V. In this mode, we wanted to differentiate between spore, vegetative bacteria, MS2 phage (virus), and ovalbumin (a toxin simulant). Our results showed that we could easily differentiate and detect the bacteria at levels of 10^3 CFU/ml with good frequency. Results with the virus and toxin were not as profound, although we were detecting the protein at 10 ng/ml about 50% of the time. Our results show that relatively simple and inexpensive cytometers with red-diode lasers as excitation sources may be an effective platform for use in a biological detector system.

INTRODUCTION

The use of cyanine dyes, for detection or identification of BW agents, with a red diode laser is of particular interest to the community since these lasers are relatively small and efficient. Since the 1990's the use of low power 670nm diode laser in products from bar code scanners to television sets to laser pointers has been wide spread, thus making red diode lasers inexpensive and a fieldable alternative to the argon laser, which is more commonly used. Additionally, the background autofluorescence of environmental aerosol particulate matter, which can be high enough to interfere

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with analysis performed with an argon laser, should not be a factor when utilizing a red diode laser. The drawback of utilizing a red diode laser is the relative lack of DNA-intercalating and other dyes that are excitable over 650nm. For this reason the use of the red-diode-excitable cyanine dyes YOYO-3, TOTO-3 and TOPRO-3 as potential reagents for BW agent detection was explored. Another reason is that this laboratory has considerable experience with the blue-excitable analogs of these dyes.

MATERIALS AND METHODS

YOYO-3 iodide is a dimer cyanine and an impermeant high -affinity DNA stain. YOYO-3 is essentially nonfluorescent in the absence of nucleic acids and is 1000 times more fluorescent when nucleic acid - bound than in free form. YOYO-3 was purchased from Molecular Probes (Y-3606, Lot: 3121-5), and diluted in water to a final concentration of 10μM.

TOTO-3 iodide is a dimer cyanine and an impermeant high -affinity DNA stain. It has absorption/emission maxima of 640/660 when bound to DNA. TOTO-3 is essentially nonfluorescent in the absence of nucleic acids and is1000 times more fluorescent when nucleic acid - bound than in free form. TOTO-3 was purchased from Molecular Probes (T-3604, Lot: 3121-9), and diluted in water to a final concentration of 10μM.

TOPRO-3 iodide is a monomer cyanine and an impermeant high -affinity DNA stain. It has absorption/emission maxima of 642/661 when bound to DNA. TOPRO-3 is essentially nonfluorescent in the absence of nucleic acids and very fluorescent when nucleic acid - bound than in free form. TOPRO-3 was purchased from Molecular Probes (T-3605, Lot: 3121-13), and diluted in water to a final concentration of 10μM.

The organisms used in this experiment were BG, *C. sporigenes*, *E.coli*, and *E. herbicola*. Each organism was grown on agar plates to verify the purity of each culture. Samples consisted of 400ul of 10^6 cfu/ml of bacteria. Several protocols were used, which included various amounts of dye; incubation times from zero to fifteen minutes; no fixative; isopropanol and ethanol as fixatives; Triton X-100, FACSLyse, and FACSperm as pre-treatments. All protocols were tested on the FACSCalibur, using the red diode laser. Samples were collected for 60 seconds or 10,000 events at various flow rates. After samples were run, the 10^6 cfu/ml dilutions of each organism was again grown on agar plates to verify the viability of the cultures.

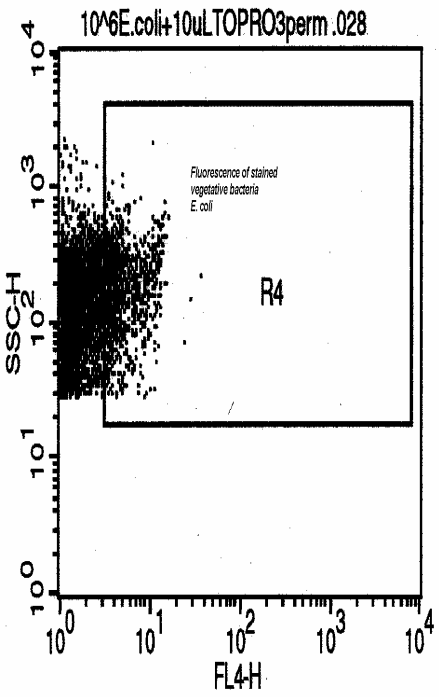
RESULTS AND DISCUSSION

YOYO-3 did not produce a shift in fluorescence, and gave no indication of staining any bacteria, under any conditions. (Data not shown.) All of the samples plated produced bacterial growth on agar plates.

TOTO-3 did not produce a shift in fluorescence, and gave no indication of staining any bacteria, under any conditions. (Data not shown.) All of the samples plated produced bacterial growth on agar plates.

Both vegetative and sporulating bacteria were detected with TOPRO-3 using little or no incubation time. Upon staining, the fluorescence of the vegetative bacteria created a distinctive pattern that was

different than the pattern produced by staining the sporulating bacteria (Figure 1). All of the samples that were plated produced bacterial growth on agar plates.



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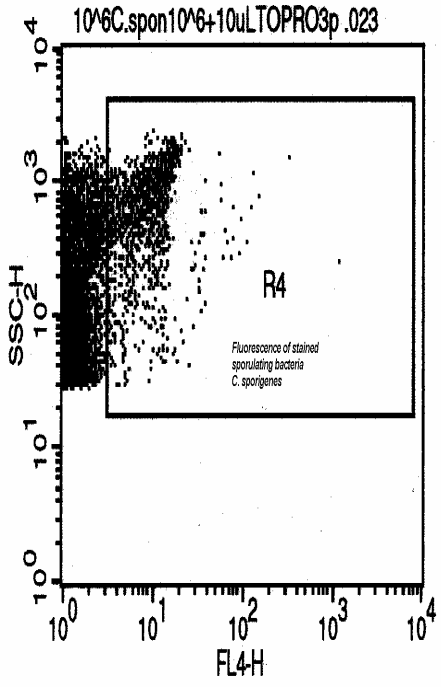

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R3	1105	15.48	15.48
R4	1110	15.55	15.55

Figure 1. Distinct patterns produced by staining vegetative and sporulating bacteria with TOPRO.

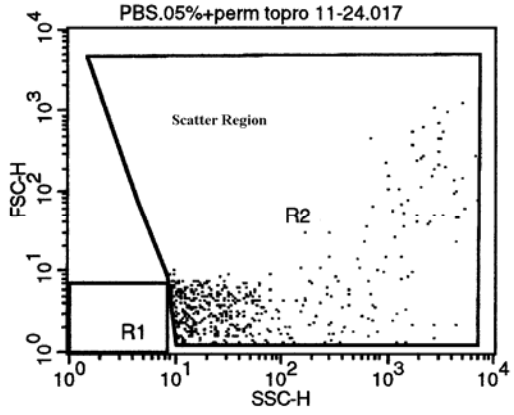
Both YOYO-3 and TOTO-3 were unable to elicit any fluorescent response when introduced to vegetative or sporulating bacteria. The molecules of these dimer cyanine dyes are probably too large to interact with the DNA of the bacteria under the restrictions of fieldable protocols. Additionally, the excitation of YOYO-3 is 612nm, which could be too low for the 635nm red diode to excite.

TOPRO-3 is a monomeric cyanine dye; thus, a much smaller molecule that either of the dimer cyanine dyes analyzed. This size difference could explain the ability of TOPRO-3 to stain both vegetative and sporulating bacteria, and may be easier to permeate the cell.

Despite the fact that TOPRO-3 is a considered as a membrane impermeable dye, both the vegetative and sporulating bacteria tested produced a shift in fluorescence without a fixative. Although the vegetative cells stained equally well with or without the fixative, the spore produced a significantly higher fluorescence when the fixative was added. Furthermore, when the vegetative cells were incubated with the dye, a significantly higher fluorescence shift was produced; however, when the vegetative cells were also incubated with the fixative, only a slight fluorescence shift was produced. Conversely, the changing of incubation times, with either the dye or the fixative, did not produce any changes in fluorescence.

The ability of TOPRO-3 to produce two different patterns presented an effective way to differentiate between spores and vegetative bacteria. Additionally, the fluorescence of the dye with diluents and ambient backgrounds was considerably lower than the fluorescence produced by any of the argon laser excitable dyes studied. The combination of this spore/vegetative differentiation and the low baseline should allow for lower levels of detection of BW agents. Therefore, this dye was investigated as a potential detector of BW simulants at JFT V.

During JFT V, each participant received a standard and then blind sets of prepared laboratory samples These were analyzed on the FACSCalibur using the red diode laser. The detection scheme was used as a proof of concept that a low power, low cost laser can provide detection. TOPRO-3, with its ability to differentiate between vegetative and sporulating bacteria at relatively low concentrations, and to detect virus and protein, was chosen as the bio-detection reagent. The optimum assay for this dye was 400µl of sample combined with 100µl of FACSPerm, and 10µl of TOPRO-3. No incubation was necessary to produce the distinct fluorescent shifts. The lack of any apparent interaction between the diluent and this dye allowed for detection at the lowest level, 10^2 cfu/mL. Regions were designated to distinguish *E. herbicola* and BG (Figure 2).



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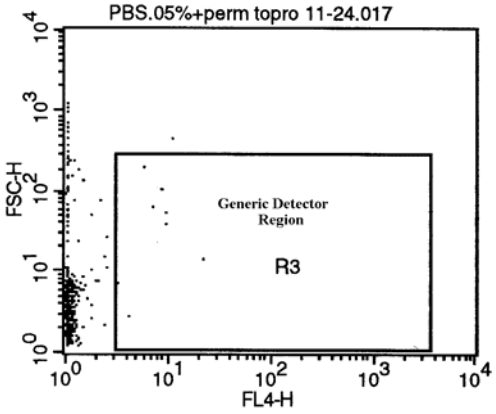
R3	8	1.69	1.69
R4	0	0.00	0.00
R2	472	100.00	100.00
R5	8	1.69	1.69



Log Data Units: Linear Values
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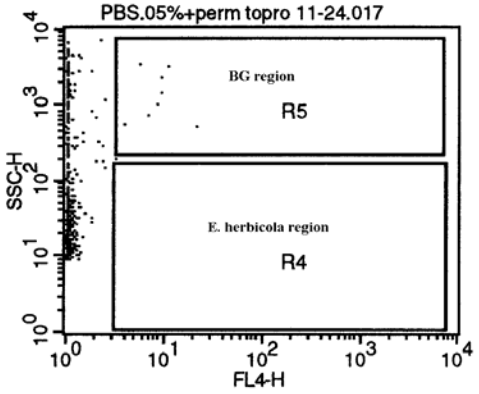
Total Events: 472

Region	Events	% Gated	% Total
R1	0	0.00	0.00
R3	8	1.69	1.69
R4	0	0.00	0.00
R2	472	100.00	100.00
R5	8	1.69	1.69



Log Data Units: Linear Values
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R1	0	0.00	0.00
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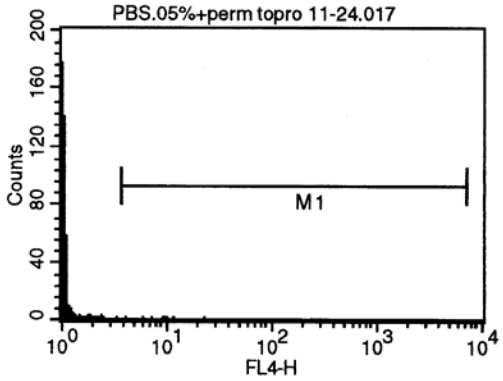
R2	472	100.00	100.00
R5	8	1.69	1.69



Log Data Units: Linear Values
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Events	% Gated	% Total	CV	Median	Peak Ch
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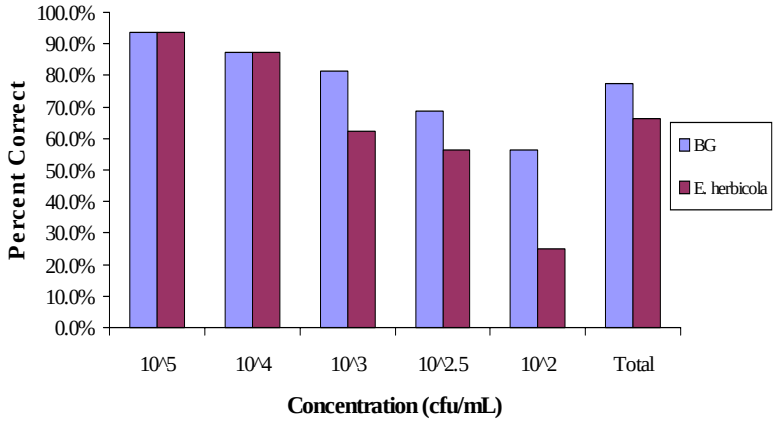
8	1.69	1.69	56.34	9.06	4

Figure 2. Sample dot plot used to distinguish BG, *E. herbicola*, MS2 and Oval.

Figure 3. JFT V Laboratory Detector Results with BG and *E. herbicola*



The use of a red diode laser with a DNA dye is a good candidate as a fieldable biodetector. TOPRO-3 produces a very low fluorescence, in the absence of antigen, and diluents and non-biological particles also produce a low autofluorescence in the presence of the red diode laser. This was verified by the correct detection of 88% of the blank samples tested. Additional testing should be initiated to determine if the added sensitivity needed to detect bacteria below 10^3 cfu/ml is worth the trade-off of sensitive assays that yield any false positives.

Not only was over 50% of both bacteria detected at 10^2.5, but this detection assay was also able to distinguish between the vegetative, *E. herbicola*, and the sporulating, BG, bacteria. MS2 virus was detected 50% of the time at 10^7 pfu/ml while Ovalbumin was detected correctly approximately 30% of the time at concentrations of 10 ng/ml or lower.

CONCLUSION

These results show that a flow cytometer equipped with a red diode laser and used in tandem with TOPRO-3 dye may be an effective platform for use in biological detection.